APPLICATION

FOR

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FOR

ASSAYS AND THERAPIES FOR LATENT VIRAL INFECTION

Background of the Invention

This is in the area of the prevention, diagnosis, and treatment of latent viral infections, such as infections with DNA viruses like Epstein-Barr virus.

10   This application claims priority to U.S.S.N. 60/167,212 filed November 24, 1999, by John B. Harley, Judith Ann James, and Kenneth M. Kaufman.

Epstein-Barr virus has been known for more than three decades. Epstein-Barr has been associated with cancer and several autoimmune diseases. Since there is evidence implicating Epstein-Barr virus in infectious

15   mononucleosis, B cell lymphoma (in immunocompromised hosts), Burkitt's lymphoma, nasopharyngeal carcinoma, and some cases of Hodgkin's lymphoma, there have been efforts to provide a vaccine against Epstein-Barr virus (Morgan, A.J., et al. J. Med. Virol. 29:74-78 (1989); Morgan, A.J. Vaccine 10:563-571 (1992); Morgan, A.J. Development of Epstein-Barr Virus Vaccines (R.G.

20   Landes Company, Austin, Texas (1995 by Springer-Verlag, Heidleberg, Germany)); Krause, P.R. & Strauss, S.E. Infect. Dis. Clin. N.A. 13:61-81 (1999)). Recombinant vectors expressing gp340/220 in a bovine papillomavirus vector or in an adenovirus vector protected five of six cottontop tamarins from lymphomas that otherwise occur after infection with Epstein-Barr virus (Finerty,

25   S., et al. J. Gen. Virol. 73:449-453 (1992)). A subunit of the gp340/200 in alum only protected three of five cotton top tamarins from lymphomas (Finerty, S., et al. Vaccine 12:1180-1184 (1994)), suggesting that this strategy might not be especially effective. A trial of an Epstein-Barr virus vaccine of gp340/220 in a Vaccinia virus vector has been reported from China and failed to protect a third

30   of those immunized from infection (Gu, S. et al. Dev. Biol. Stand. 84:171-177 (1995)), consistent with the primate data. Khanna, et al., J. Immunol. 162:3063-3069 (1999) reports on the potential use of gp350/gp85 CTL epitopes in vaccine design to protect against EBV. However, as they note, evidence of neutralizing antibodies does not always correlate with protection against infection. Their

35   research is focused on structural antigens, gp350 and gp85, from which they

5 synthesized specific peptides to use as vaccines. They observed higher CTL

reactivity to lytic antigens as compared to a latent antigen, LMP-1. Jackman, et

al., _Vaccine_ 17:660-668 (1999) also reported on studies using gp350. Gu, et al.,

Dev. Biol. Stand. 84:171-177 (1995) reports on a clinical trial based on live

recombinant vaccinia virus expression gp350 as an immunogen, but there were

10 safety concerns.

Another method of vaccine development has been considered. Cytotoxic

T cell epitopes have been mapped for several different EBV antigens in persons

of specific HLA haplotypes. For example, trials are underway to evaluate the

usefulness of immunization with an EBNA-3 peptide which is a dominant CTL

15 epitope in HLA-B8 restricted persons (Schmidt, et al. _Proc. Natl. Acad. Sci._

USA 88:9478-82 (1991)). Efficacy of these vaccines is currently unknown.

A variety of additional therapies against Epstein-Barr virus have been

attempted. These include inducing the lytic cycle in cells latently infected by

virus (Gutierrez, M.I., et al. _Cancer Res._ 56:969-972 (1996)). Patients with the

20 Epstein-Barr virus related lymphomatoid granulomatosis have been treated with

interferon-alpha 2b (Wilson, W.H., et al. _Blood_ 87:4531-4537 (1996)).

Cycloheximide and acycloguanosine have been demonstrated to be useful _in_

vitro (Ishii, H.H., et al. _Immunol. Cell Biol._ 73:463-468 (1995)); however, only

limited further evaluation of this therapy has proceeded due to the limited

25 clinical benefit of acycloguanosine in primary EBV infection.

Therapy with a T cell line has been attempted (Kimura, H. et al. _Clin._

Exp. Immunol. 103:192-298 (1996)), as has adoptive transfer of gene-modified

virus-specific T lymphocytes (Heslop, H.E. et al. _Nature Med._ 2:551-555

(1996)). Data available do not appear to particularly support the use of

30 acyclovir for Epstein-Barr virus infections (Wagstaff, A.J., et al. _Drugs_ 47:153-

205 (1994)), though FK506 (a relative of cyclosporine) may have some benefit

(Singh, N., et al. _Digestive Dis. Sci._ 39:15-18 (1994)). Monoclonal antibodies

have been used to treat the virus-induced lymphoproliferative syndrome

(Lazarovots, A.I., et al. _Clin. Invest. Med._ 17:621-625 (1994)) with modest early

35 success.

5   Therapeutic strategies directed against the latent phase of the viral

infection have been considered. Among these is the consideration of the use of

T cell epitopes of particular HLA-restricted cytotoxic T cells (e.g. see Morgan,

A.J. Development of Epstein-Barr Virus Vaccines (R.G. Landes Company,

Austin, Texas (1995 by Springer-Verlag, Heidleberg, Germany), pp109-115; or

10   Barnes J. Pharma Weekly 1:11 (1995)). The latent membrane proteins have not

been shown to be accessible on the outside of the cell.

Epstein-Barr virus is most similar to the other members of the

Herpesviridae family. Although only gamma herpesvirus which infects humans,

all human herpesviruses have linear double-stranded DNA, an icosadeltahedral

15   capsid, a tegument which surrounds the capsid and an envelope containing viral

glycoprotein spikes on its surface. All human herpesviruses remain with and

survive in the host after primary infection.

Cytomegalovirus, in a manner similar to Epstein-Barr virus, is able to

establish latency in peripheral immune cells; however, the exact cellular location

20   is still in dispute (Mocarski, E.S. "Cytomegaloviruses and their replication"

Fields Virology, third edition (eds. B.N. Fields, et al., Lippincott-Raven (1996)),

2447-2480). Human CMV has been treated with a host of antiviral agents,

including leukocyte interferon, interferon stimulators, transfer factor, acyclovir

and nucleoside inhibitors, as well as combination therapy with interferon and

25   ara-A (reviewed by Alford, C.A. Antiviral agents and viral diseases of man 2nd

ed. New York: Raven Press; 433-86 (1984); Ho, M. Cytomegalovirus, biology

and infection: current topics in infectious disease New York: Plenum Press; 105-

18 (1982)). Very little to no clinical benefit has been demonstrated with these

therapies and overwhelming toxicities are present (Alford, C.A. Antiviral agents

30   and viral diseases of man 2nd ed. New York: Raven Press; 433-86 (1984)).

Gancyclovir (through its nucleic acid chain-terminating activity) and

foscarnet (through inhibition of viral DNA polymerase directly) have both been

used with success (Snoeck, R., Neyts, J., De Clerq, E. Multidisciplinary

approach to understanding cytomegalovirus disease Amsterdam: Excerpta

35   Medica: 1993, 269-78). Both of these drugs have been used with some success

5   in the prophylaxis of invasive CMV in the post-transplant setting. However, drug toxicity and no evident decrease in overall mortality limits their standard use for all patients (Goodrich, J.M, et al. Ann. Intern. Med. 118:173-8 (1993); Reusser P, et al. J. Infect. Dis. 166:473-79 (1992)).

Passive immunoprophylaxis remains very controversial. Newer drugs

10   targeting the CMV protease and DNA processivity activity are under development (Digard, P., Chow, C.S., Pirrit, L., Coen, D.M. J. Virol. 67:1159-68 (1993); Ertl, P.F., Powell, K.L. J. Virol. 66:4126-33 (1992)).

Vaccine development for CMV has met with only limited success. Initial studies were performed using an attenuated laboratory strain of CMV (Elek,

15   S.D., Stern, H. Lancet 1:1-5 (1974); Plotkin, S.A., et al. Infect. Immun. 12:521-27 (1975)). These British studies showed limited immunity which decreased with time (Plotkin, S.A. , et al. J. Infect. Dis. 159:860-65 (1989)). Safety issues concerning immunization of slightly immunocompromized subjects and women of childbearing age have also limited excitement over these studies. Subunit

20   vaccines for CMV prevention are also under consideration. Early studies with the major envelope glycoprotein, gB, are under way (Plotkin, S.A., et al. Rev. Infect. Dis. 12:827-38 (1990);Spaete, R.R., Transplant Proc. 23:90-96 (1991)) and show some early promise.

Human alphaherpesviruses, including herpes simplex-1, herpes simplex-

25   2, varicella-zoster and human herpesvirus 8, primarily establish latency in the sensory ganglia. Substantial evidence is present for the chemotherapy of the herpes-simplex viruses with specific anti-viral therapy, such as acyclovir, gancyclovir and forcarnet (reviewed Rickinson, A.B., Kieff E., "Epstein-Barr virus", Fields Virology 2397-2446 (1996)). Numerous vaccines for HSV-1 and

30   HSV-2 have been developed with poor success (reviewed by Whitley, R.J. "Herpes Simplex viruses", Field's Virology 2297-2330 (1996)). Two new approaches are of interest. One is based upon the production of adequate amounts of recombinant HSV-2 glycoproteins B or D to be used either separately or together as a subunit vaccine (Ashley, R., Mertz, G.J., Corey, L. J.

35   Virol. 61:253-8 (1987); Zarling, J.M., et al. J. Virol. 62:4481-85 (1988)).

5   Concerns with these studies include the use of Freund's adjuvant or a lipophilic

muramyl tripeptide, neither of which is acceptable human adjuvants. The second

method is to genetically engineer a live, attenuated recombinant HSV that

combines type 1 and type 2 genomes without the putative neurovirulence

sequences (Meignier, B., Longnecker, R., Roizman, B. J. Infect. Dis. 162:313-

10   21 (1990)).

Varicella zoster also establishes latency in sensory neurons. This virus in

its typical primary infection of the juvenile host is only treated with supportive

measures with full recovery usually within one week. However, life-threatening

primary and recurrent infections are encountered in immunocompromized hosts.

15   These overwhelming infections are usually treated with acyclovir. Gancyclovir

and famcylovir are used for treatment of resistant strains. A live, attenuated

varicella vaccine is in common use; however, long-term efficacy studies for this

vaccine are still pending.

The human herpesvirus-8 is the other alpha herpesvirus. Only recently

20   identified and characterized, neither therapy trials nor vaccine development has

yet begun.

The problem within latent viral infections is the ability to identify, target,

and treat cells harboring latent virus. For example, the Epstein-Barr virus

produces certain proteins during the latent stage of its life cycle. These proteins

25   have not been shown to be antigenic in an expression system that would enable

immune system recognition. One of these proteins is LMP-2A. Antibodies

have been raised against LPM-2A and immunofluoresence used to show that

LMP-2A is located in or near the membrane (Longnecker, R. and Kieff, E., J.

Virol. 64:3219-2326 (1990)). These workers established that their antiserum

30   bound to LMP-2A isolated from the cell, but it was not established that the

extracellular part of LMP-2A was antigenic, i.e., could elicit antibody

production or was accessible to be bound by antibody (or any other external

ligand). In fact, these workers concluded that their antibodies recognized the

intracellular portion of LMP-2A because the antibodies were LMP-2A specific

35   and it is the LMP-2A intracellular region which differentiates LMP-2A from

5   LMP-2B. (Actually, these two Epstein-Barr virus proteins are in large part

identical and appear to vary because splice junction variation gives LMP-2A a

longer amino terminal cytoplasmic tail than is found in LMP-2B.)

It is therefore an object of the present invention to provide methods to

target cells that are infected with a virus, such as a DNA virus like a herpes

10   virus, in the latent stage of its life cycle.

It is a further object of the present invention to provide strategies to

target cells containing Epstein-Barr virus in a latent state.

It is another object of the present invention to provide strategies to treat

or prevent diseases linked to these latent viruses.

15   It is another object of the present invention to provide vaccines based

upon the structure of proteins expressed during the latent life cycle of a virus,

such as Epstein-Barr virus.

It is a further object of the present invention to provide methods for the

development of therapeutics to treat diseases linked to Epstein-Barr virus by

20   targeting cells latently infected with Epstein-Barr virus.

It is a further object of this invention to provide diagnostics which will

identify people with latent viral infections, such as infections with Epstein-Barr

virus.

It is a further object of this invention to provide diagnostic tests which

25   will help distinguish those with a disease from those without a disease by

differences in the immune responses to a DNA virus, such as Epstein-Barr virus.

Summary of the Invention

Compositions that bind viral proteins that are specifically expressed

30   during the latent stage of the viral life cycle are disclosed. These compositions

bind the latent viral proteins while the viral proteins are expressed in their

cellular host, and provide a means for targeting cells that harbor latent virus. In

a preferred embodiment the compositions are antibodies which bind the

extracellular region of the latent viral protein, most preferably LMP-2A, an EBV

35   latent protein, which are conjugated to a diagnostic or cytotoxic agent or

5   immobilized to a solid support for removal of the infected cells. These

antibodies are capable of distinguishing cells expressing EBV DNA from cells

which are not expressing EBV DNA. Compositions that can be used to elicit

production of these antibodies, or as a vaccine, are also disclosed.

Methods for generating diagnostic or cytotoxic reagents and vaccines

10   based on the viral epitopes that identify cells harboring latent virus are also

disclosed.

The antibody conjugates can be used in diagnostic assays to identify

cells expressing latent viral protein and people who are harboring latent viral

particles. The antibody conjugates can also be used to remove the infected cells

15   or to kill the infected the cells. Alternatively, or in addition, the viral proteins or

portions thereof can be used as a vaccine to induce an immune reaction by the

host to kill the infected cells. These methods can be used to detect or treat

patients harboring latent viruses like EBV and who are at risk of developing a

disease such as an autoimmune disease like systemic lupus erythematosus (SLE)

20   and rheumatoid arthritis (RA).

Detailed Description of the Invention

Definitions

As used herein, autoimmune diseases are diseases that are primarily

autoimmune, as well as diseases which do not appear to be primarily

25   autoimmune but have immune manifestations involving immunoglobulins,

antigen specific B cell surface receptors (surface immunoglobulin), or antigen-

specific T cell receptors. Examples of diseases which fall into these categories

are systemic lupus erythematosus, Sjogren's syndrome, rheumatoid arthritis,

juvenile onset diabetes mellitus, Wegener's granulomatosis, inflammatory bowel

30   disease, polymyositis, dermatomyositis, multiple endocrine failure, Schmidt's

syndrome, autoimmune uveitis, Addison's disease, adrenalitis, primary biliary

cirrhosis, Graves' disease, thyroiditis, Hashimoto's thyroiditis, autoimmune

thyroid disease, pernicious anemia, gastric atrophy, chronic hepatitis, lupoid

hepatitis, atherosclerosis, presenile dementia, demyelating diseases, multiple

35   sclerosis, subacute cutaneous lupus erythematosus, hypoparathyroidism,

5   Dressler's syndrome, myasthenia gravis, autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura, hemolytic anemia, autoimmune hemolytic anemia, pemphigus vulgaris, pemphigus, bullous pemphigoid, dermatitis herpetiformis, alopecia areata, autoimmune cystitis, pemphigoid, scleroderma, progressive systemic sclerosis, CREST syndrome (calcinosis, Raynaud's

10   esophageal dysmotility, sclerodactyly, and telangiectasia), adult onset diabetes mellitus (Type II diabetes), male or female autoimmune infertility, ankylosing spondylitis, ulcerative colitis, Crohn's disease, mixed connective tissue disease, polyarteritis nodosa, systemic necrotizing vasculitis, juvenile onset rheumatoid arthritis, glomerulonephritis, atopic dermatitis, atopic rhinitis, Goodpasture's

15   syndrome, Chagas' disease, sarcoidosis, rheumatic fever, asthma, recurrent abortion, anti-phospholipid syndrome, farmer's lung, erythema multiforme, postcardotomy syndrome, Cushing's syndrome, autoimmune chronic active hepatitis, bird-fancier's lung, asthma, allergic disease, allergic encephalomyelitis, toxic necrodermal lysis, alopecia, Alport's syndrome,

20   alveolitis, allergic alveolitis, fibrosing alveolitis, interstitial lung disease, erythema nodosum, pyoderma gangrenosum, transfusion reaction, leprosy, malaria, leishmaniasis, trypanosomiasis, chronic fatigue syndrome, fibromyalgia, Takayasu's arteritis, Kawasaki's disease, polymyalgia rheumatica, temporal arteritis, schistosomiasis, giant cell arteritis, ascariasis, aspergillosis,

25   Sampter's syndrome (triaditis also called, nasal polyps, eosinophilia, and asthma), Behcet's disease, Caplan's syndrome, dengue, encephalomyositis, endocarditis, myocarditis, endomyocardial fibrosis, endophthalmitis, erythema elevatum et diutinum, psoriasis, erythroblastosis fetalis, fascitis with eosinophilia, Shulman's syndrome, Felty's syndrome, filariasis, cyclitis, chronic

30   cyclitis, heterochromic cyclitis, Fuch's cyclitis, IgA nephropathy, Henoch-Schonlein purpura, glomerulonephritis, graft versus host disease, transplantation rejection, cardiomyopathy, Alzheimer's disease, parvovirus infection, rubella virus infection, post vaccination syndromes, congenital rubella infection, Hodgkin's and non-Hodgkin's lymphoma, renal cell carcinoma, multiple

35   myeloma, Eaton-Lambert syndrome, relapsing polychondritis, malignant

5   melanoma, cryoglobulinemia, hepatitis B virus infection, hepatitis C virus

infection, Waldenstrom's macroglobulinemia, mumps virus infection,

thrombotic throbocytopenic purpura and any other disorder in which the specific

recognition of the host by immunoglobulin, B cell surface receptor (surface

immunoglobulin), or T cell receptor is suspected or shown to be important in

10   any aspect of the pathogenesis of the clinical illness.

Immunodeficiency is any reduction in humoral and/or cellular immunity

of the normal young adult level which decreases the capacity to protect from

infection or respond to an immunogen. Examples of immunodeficiency include

infections (tuberculosis, human immunodeficiency virus (HIV) infection,

15   malaria, parasitic infections and a host of others), extremes of age (the fetus,

neonates, young children (less than 2 years of age), and old age (over 65 years

of age), trauma, severe anemia, burns, exposure, nutritional deficiencies

(nutritional, vitamin, starvation, etc.), cancers, hereditary, acquired

immunodeficiences due to medications (i.e. transplantation patients,

20   chemotherapy patients, etc.), immune deficiencies (of immunoglobulin, T cells,

any number of other genetic defects, etc.) and idiopathic diseases with acquired

immune deficiency (sarcoidosis, rheumatoid arthritis, systemic lupus

erythematosus, etc.).

Immunization is any procedure which leads to a cellular or humoral

25   immune response directed against an identifiable and specific antigen, usually

the immunogen. An antigen is a substance that is bound by antibody or leads to

a T cell mediated immune response.

An autoantigen is a constituent of self that binds antibody (making it an

autoantibody) or that induces a cellular response, for example, by a T cell. The

30   cellular response may be assayed by presentation of a peptide from the

autoantigen, proliferation, cell activation, the prevention of cell activation,

secretion of cytokines, activation of apoptosis, or other indication of an effect of

the presence of the autoantigen.

An autoantibody is any immunoglobulin, antigen specific B cell surface

35   receptor (surface immunoglobulin), or antigen specific T cell receptor directed

5   against self-protein, carbohydrate or nucleic acid. Such T cell receptors usually bind peptides which themselves are bound by histocompatability molecules. The T cell receptor usually binds to both the peptide and the histocompatibility molecule.

Therapy is a treatment by medical or physical means. A "treatment" is

10   the composition used for treating a condition. Antiviral therapy is the use of a treatment in an effort to suppress or eliminate a virus, for example, suppression, elimination or other amelioration of the effect of Epstein-Barr virus.

Peptides are small proteins composed of amino acids, typically at least four to seven amino acids in length, covalently bound to one another by peptide

15   bonds. Peptides may be prepared by an *in vivo* mechanism, as in life, by using the nucleic acid encoding for the sequence of the peptide produced, or *in vitro* using peptide chemistry. A vaccine is a composition including antigen that elicits an immune response effective to prevent or ameliorate a disease.

Seroconversion means that the subject has developed antibodies to an

20   immunogen. Usually, this is the result of immunization (vaccination) or infection. Seropositive means that there are a sufficient quantity of antibodies with sufficient affinity to conclude that seroconversion has occurred. Seronegative means that the quantity and affinity of antibodies are not sufficient to conclude that seroconversion has occurred. In this application, the terms

25   "lupus" and "systemic lupus erythematosus" are used interchangeably.

The term linear epitope as defined by a specific sequence is used herein to include peptides having substitutions yielding a peptide bound in an equivalent manner or extent by an antibody or autoantibody.

I.   Compositions for Diagnosis and Treatment of Latent Infections

30   Compositions have been prepared based on information regarding unique epitopes of molecules specific to latently infected cells. This is exemplified in the attached examples. In general, viral proteins, expressed only on the surface of latently infected cells, are identified and antibodies prepared which are reactive with the extracellular portion of the proteins. These antibodies may be

35   prepared using isolated antigen, followed by screening for reactivity with

5   latently infected cells, or by immunization with an extracellular fragment

(prepared by enzymatic cleavage or recombinant techniques), alone or in

combination with an adjuvant or as a fusion protein. The antibodies can be used

as whole antibodies, antibody fragments, labeled with diagnostic labels, or

bound to a cytotoxic agent to kill infected cells. Alternatively, the antigens can

10   be used to prepare vaccines which are used to immunize an individual who has,

or is at risk of, a latent viral infection.

Viruses

There are a number of different classes of viruses. There are class I and

class II DNA viruses, differentiated by the fact that class I viruses contain

15   nucleic acid in a double-stranded genome and class II viruses contain nucleic

acid in a single-stranded genome. Adenoviruses, Herpesviruses, SV40 viruses,

and Vaccinia viruses are all class I and Parvoviruses are class II viruses. Classes

III (reovirus), IV (poliovirus), V (vesicular stomatitis virus), and VI

(retroviruses) are RNA viruses.

20   Some viruses have two stages: (1) when the virus is infectious, the lytic

stage, and (2) when the virus can persist in the host, the latent stage.

The lytic stage produces new viral particles, which are infectious.

During the lytic stage the viral gene products expressed are sufficient to

commandeer the cellular machinery to make new viral particles. When a virus

25   is in a lytic stage, the amount of viral material ensures that nearly all infected

individuals will develop an immune response to at least some part of the virus.

Consequently, a vigorous immune response against the virus and the virus

producing host cells usually develops when the virus is in the lytic stage.

During the latent stage the virus expresses only a few of its gene

30   products and its genetic material is passed to daughter cells during cell division.

Since only small amounts of very few proteins are expressed from the viral

genome, and since there are specific mechanisms in latency to avoid immune

recognition or to inhibit anti-viral immunity, the immune response to viral

material which is produced during some the latent stages of the life cycle is

5   small or non-existent. Consequently, the virus can survive in the infected host

for many years. The host most often remains infected for the duration of its life.

Infrequently, a latent virus can become "activated" and the infections

become lytic. New virus is then made and the infection is spread in the host,

unless the immune response of the host intervenes to prevent the successful

10   production of new virus. This process typically occurs and reoccurs throughout

the life of the host.

Epstein-Barr Virus

Epstein-Barr virus is a herpes virus, Human Herpes Virus 4 (HHV-4).

This virus is from the genus *Lymphocryptovirus* and subfamily

15   gammaherpesvirinae. This is the only gamma herpes virus known in man. Like

the other herpes viruses, this is a DNA virus and has a strong propensity for

latency. Once latent this virus emerges from latency at a low level throughout

life.

Epstein-Barr virus infects B cells and induces a large number of different

20   antibodies in the early stage of infection. In most people the B cell proliferation

and antibody production is eventually brought under control by virus specific T

cells. Thereafter, the virus remains latent, surviving in the host for the

remainder of the natural life. The virus continues to "reactivate" at a low level,

as evidenced by the shedding of virus in the oral cavity, infection through

25   exchange of oral secretions, the spontaneous *in vitro* outgrowth of transformed

B cells, and the spontaneous production of Epstein-Barr virus *in vitro*. The

continuous presence of virus presents a significant challenge to the immune

system and requires that the immune mechanisms sustain viral suppression over

the many decades of remaining life. The immune system recognizes the

30   antigens of the lytic stage (and whole virus) much better than the latent stage.

Most of the T cell responses are directed against lytic stage antigens and may

tend to concentrate on the antigen known as EBNA-3A (Epstein-Barr nuclear

antigen-3A). CD8 T cell responses against the latent stage antigen ENBA-1 are

specifically inhibited, even though antibody is frequently made against this

5   antigen. This effect appears to be caused by inhibition in antigen presentation

(Levitskaya J., et al. Nature 375:685-88 (1995)).

Three different types of latency have been identified in what are now

classic studies: Type 1, Type 2, and Type 3. About eight EBV gene products are

known to be expressed in different stages of latency. In Type 1 latency, only

10   EBNA-1 is expressed. In Type 2 latency, LMP-1, LMP-2A and LMP-2B are

expressed. These three plus LP, EBNA-1, EBNA-2, EBNA-3A, -3B, and -3C

are expressed in Type 3 latency. EBER RNAs may also be expressed in latency.

These three forms of latency generally conform to what has been found in

Burkitt's lymphoma (in vivo and in cell lines), some lymphomas,

15   nasopharyngeal carcinoma, and transformed B cell lines derived in vitro from

normal donors.

Another latent stage has recently been described in which only a single

gene, latent membrane protein 2A (or LMP-2A), is expressed (Miyashita, E. J.

Virol. 71:4882-91 (1997)). The amino acid sequence and DNA sequence of

20   Epstein-Barr virus LMP-2A is found at Swiss Protein DataBank accession

number P13285 and GenBank accession number M24212. This form may

dominate in normal individuals. A form of latency observed in

immunosuppressed patients appears to consist of only EBV DNA with no LMP-

2A nor EBNA-1 expression (Babcock G, et al. J. Exp. Med., 190; 567-576

25   (1999)).

It has been asserted by some that only B lymphocytes are able to

generate productive infectious virus (Thorley-Lawson, D.A., Miyashita, E.M.,

Khan, G., Trends Micro. 4:204-8 (1996)). Indeed, some argue that only B cells

are infected in the latent state (Mikashita, E.M., et al., J. Virol. 71:4882-4891

30   (1997)). Recent observations by Thorley-Lawson show that multiple forms of

latency co-exist in different cells in the same human subject (Babcock G, et al. J.

Exp. Med., 190; 567-576 (1999)).

There are also data showing evidence for the expression of Epstein-Barr

virus genes in rheumatoid synovium (Scotet E., et al. J. Exp. Med. 184:1771-80

5   (1996)) and in the synovial cells or fibroblasts from these patients (Koide J., et

al. J. Virol. 71:2478-81 (1997); Takei M., et al., Int. Immunol. 9:739-43 (1997)).

It is believed that rheumatoid arthritis may be caused by a dysregulated

form of Epstein-Barr virus infection in the synovium. This may involve more

genes than just the known latent genes (EBERs, EBNAs, BART, LP, LMPs).

10   While no one has established whether that intact virus is produced, it is thought

that the infection is less efficient relative to a normal infection in B cells. This is

referred to as an "abortive" or "partial" or "incomplete" EBV infection. The

genes expressed include, among others, BZLF1, BMLF1, BCRF1, BMRF1,

BALF4, EBNA3A, EBNA3B, EBNA3C, and LMP2. (Scotet et al Eur J.

15   Immunol. 29:973-85 (1999)). The T cell immune response in RA synovium

appears to be strongest against a few genes involved in signaling the virus to

switch from latency to lytic infection.

Other Latent Viruses

Latency in herpes simplex virus-1 and herpes simplex virus-2 is

20   maintained with the latency associated transcripts (LAT). The major open

reading frame contains 2 kb of viral DNA. Though CD8+ T cells appear to be

important in maintaining latency, the antigen and their relation to LAT is not

known. Indeed, the protein products from the LAT genes have never been

isolated. An immune response directed against LAT RNA or LAT encoded

25   protein is not known.

In *varicella zoster* virus, the latently expressed proteins include IE4,

IE62, IE63 and ORF29p. IE63 is abundant and antigenic (Straus, S.E., et al.

Ann. Int. Med. 108:221 (1988); Donahue, J.G. et al. Arch. Int. Med. 155:1605-9

(1995); Debras, S. et al. J. Virol. 69:3240 (1995); Mahalingham, R., et al. Proc.

30   Nat'l. Acad. Sci., U.S.A. 93:2122 (1996); Sadzot-Delvaux, C., et al. J. Immunol.

159:2802 (1997); Sadzot-Delvaux, C. & Bentier, B., 24th International

Herpesvirus Workshop July 17-23 23 (Mass. Inst. Tech.) abst. 9.009 (1999)).

There is much less now known about the latent state of infection with

cytomegalovirus than with Epstein-Barr virus. Nevertheless, in both man and

35   mouse a number of genes are expressed in latent cytomegalovirus infection

5   including ORF42, ORF45, ORF55, ORF59, ORF94, ORF152, and ORF154. Of

these, at least ORF152 has a predicted transmembrane domain and may be a

membrane protein, and hence have a potential to be a target for therapeutics in a

way similar to LMP-2A, as described herein. Cytomegalovirus is a β

herpesvirus, as are HHV-6 and HHV-7. It is thought that HHV-6 ORF160 and

10   HHV-7 ORF97 are the latent homologues of HCMV ORF152 (Kondo, K. &

Yamanishi, K. 24th International Herpesvirus Workshop, July 17-23 (Mass. Inst.

Tech.) Abst. 1.016 (1999)). ORF152 has been shown to be antigenic (Kondo,

K. et al Proc. Natl. Acad. Sci. USA 93;11137-42 (1996)), but not whether the

antibodies to ORF 152 will bind the surface of cells latently infected with

15   cytomegalovirus.

Kaposi's sarcoma herpesvirus or HHV-8, another oncogenic human

gamma herpesvirus, expresses a latent protein called latency associated nuclear

antigen ("LANA" or ORF73). LANA has been shown to attach cellular DNA to

the episome of Kaposi's sarcoma herpes virus. In addition, ORF71 and ORF72

20   are expressed in the latent form of HHV-8 (Dittmer D, et al. J. Virol. 72:8309

(1998)). Both the Kaposi's sarcoma-associated herpesvirus (Human herpesvirus

8) and *Herpesvirus papio* produce latent membrane proteins similar to EBV

LMP-2A (Genbank accession # AAD45297 and accession #AAC54552

respectively). In addition the *Herpesvirus papio* also contains a latent

25   membrane protein similar to EBV LMP-1 (GenBank accession #AAB37764).

Diagnostic and Cytotoxic Agents

The antibodies or fragments thereof can be used to identify, remove

and/or kill latently infected cells. Typically, the antibody will be coupled to a

label which is detectable or cytotoxic but which does not interfere with binding

30   to the infected cells. Examples include radioisotopes, such as indium ("In"),

which is useful for diagnostic purposes, and yttrium ("Y"), which is cytotoxic.

Other detectable labels include enzymes, radioisotopes, fluorescent compounds,

chemiluminescent compounds, and bioluminescent compounds and compounds

detectable by ultrasound, MRI or CT. Other cytotoxic agents include toxins

35   such as ricin, mitomycin C, danorubicin, and vinblastin.

5   *Antibodies to the Latent Viral Proteins*

           When an antigen is presented to an immune system there are usually many antibodies produced, often recognizing different portions of the antigen. The sera of the antigen challenged animal may contain all or many of these antibodies, referred to as polyclonal antibodies. Each of the antibodies are

10   produced from a single B cell which in turn makes many clonal copies of itself. These B cells can be made into hybridomas which produce a monoclonal antibody.

           Antigens have regions called epitopes which make up the specific molecular determinants for antibody:antigen binding. Typically an epitope of a

15   protein is composed of between three or four to eight amino acids (see Watson et al., "Certain Properties Make Substances Antigenic," in <u>Molecular Biology of the Gene</u>; Fourth Edition, page 836, paragraph 3, (The Benjamin/Cummings Publishing Company, Menlo Park, 1987)). The antigens that are determinative of the latent stage of a viral infection can contain the entire native epitope, or

20   portions thereof, sufficient to react with antibody.

           An antibody to an antigen of choice can be produced in mice according to Kohler and Milstein, <u>Nature</u>, 256:495-497 (1975) and <u>Eur. J. Immunol</u>. 6:511-519 (1976), both of which are hereby incorporated by reference, by immunizing a host with the antigen of choice. Once a host is immunized with the antigen, B-

25   lymphocytes that recognize the antigen are stimulated to grow and produce antibody to the antigen. Each activated B-cell produces clones which in turn produce the monoclonal antibody. B-cells cannot be cultured indefinitely, however, so hybridomas are produced using the methods developed by Kohler and Milstein, <u>Nature</u>, 256:495-497 (1975). The antibodies produced and

30   isolated by this method are specific for a single antigen or epitope on an antigen, and are referred to as monoclonal antibodies.

           A cell bound enzyme linked immmunosorbent assay (ELISA) can be used to screen supernatants from growing hybridomas (Glassy, M. C. and Surh, C. D., <u>J. Immunol. Method</u>, 81:115 (1985)). Cells which bind the antibody or

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5   produce the antibody can be analyzed using Flow Cytometry. Cell surface
        antigens are detectable by flow cytometry.

                Solid phase binding of antibodies to peptides has proven useful for
        examining sequential linear epitopes, also referred to as "linear" or "sequential".
        These have been useful to define important residues in epitope structure. This
10   approach may or may not be less useful in defining conformational epitopes or
        for defining regions where two or more linear, but not sequential, epitopes are
        brought together by the tertiary structure. The particular structural relationships
        between the antigens derived from viral proteins expressed during the latent life
        cycle of a DNA virus and the antibodies that bind them with particular regard to
15   the specific conformation assumed by the peptide determines what can be
        learned by this approach. In addition, although many peptides may assume
        conformations in solution that are not found in the native protein structure, true
        epitopes may still be delineated by this method. Those peptides that tend to
        have a structure similar to that found in the native molecule are usually expected
20   to be bound by a larger proportion of the antibodies that bind the analogous
        sequence on the native protein and/or may be bound with greater affinity.

                Useful latent viral proteins to make antibodies for use in the conjugates
        disclosed herein are described above. Proteins that are expressed on the cell
        surface of the cells harboring the latent DNA virus are preferred. A preferred
25   latent viral protein is the LMP-2A protein of EBV. Vaccines including this
        protein, and more preferably the extracellular portion of this protein, and
        optionally an adjuvant, can be used to immunize patients against the latent
        infection, or for this application, to make antibodies for use as conjugates.
        Suitable antibodies can be produced as described in example 1. Though
30   antibodies against the latent membrane proteins have long been appreciated (e.g.
        Rowe, M. et al., J. Gen. Virol. 69:1217-28 (1988)), including a remarkably high
        frequency of antibodies against LMP-2A and LMP-2B in nasopharyngeal
        carcinoma (Lennette, E.T. et al., Eur. J. Cancer 31A:1875-8 (1995)), the
        presence of antibody binding to LMP-2A or LMP-2B from the external cell
35   surface has not been demonstrated previously.

5   LMP-1 is also expressed during some phases of latency. Depending upon their expression patterns, this membrane protein may also be a useful target for anti-EBV therapeutics in the EBV related diseases, much as LMP-2A appears to be and LMP-2B are.

Other viral proteins of interest include cytomegalovirus (CMV), Kaposi,
10   and other herpes viruses, especially human herpes virus 6, varicella 3, and CMV 5.

While the *in vivo* use of a monoclonal antibody from a foreign donor species in a different host recipient species is usually uncomplicated, an antigenic site on the donor antibody can cause an adverse immunological
15   response in the organism receiving the donor antibody. The adverse response may serve to hinder the molecular interaction of the donor antibody or acceptance of the donor antibody. Chimeric antibodies can be used to reduce or eliminate the adverse host response (Sun, L.K., et al., Hybridoma, 5 (Supplement 1):S17 (1986); Oi, et al., Bio Techniques, 4(3): 214 (1986)).
20   Chimeric antibodies are antibodies in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species. Typically, a chimeric antibody will comprise the variable domains of the heavy (V_H) and light (V_L) chains derived from the donor species producing the antibody of desired antigenic specificity, and the variable domains of the
25   heavy (C_H) and light (C_L) chains derived from the host recipient species. It is believed that by reducing the exposure of the host immune system to the antigenic determinants of the donor antibody domains, especially those in the C_H region, the possibility of an adverse immunological response occurring in the recipient species will be reduced. Thus, for example, it is possible to produce a
30   chimeric antibody for *in vivo* clinical use in humans which comprises mouse V_H and V_L domains coded for by DNA isolated from a rabbit that binds LMP-2A and C_H and C_L domains coded for with DNA isolated from a human immune system cell.

Under certain circumstances, monoclonal antibodies of one isotype
35   might be more preferable than those of another in terms of their diagnostic or

5   therapeutic efficacy. For example, from studies on antibody-mediated cytolysis,
it is known that unmodified mouse monoclonal antibodies of isotype gamma-2a
and gamma-3 are generally more effective in lysing target cells than are
antibodies of the gamma-1 isotype. This differential efficacy is thought to be
due to the ability of the gamma-2a and gamma-3 isotypes to more actively

10   participate in the cytolytic destruction of target cells. Particular isotypes of a
monoclonal antibody can be prepared either directly, by selecting from the
initial fusion, or prepared secondarily, from a parental hybridoma secreting a
monoclonal antibody of different isotype by using the sib selection technique to
isolate class-switch variants (Steplewski, et al., Proc. Natl. Acad. Sci., U.S.A.,

15   82:8653 (1985); Spira, et al., J. Immunol. Meth., 74:307 (1984)). Thus, the
antibodies described herein include class-switch variants having the specificity
of monoclonal antibodies derived to the extracellular regions of LMP-2A.

Antibody fragments, such as, for example, Fab and $F(ab')_2$, as well as
isotypes, particularly if labeled or bound to a cytotoxic agent, can be used since

20   having an effect on the viral infection in these situations is not dependent upon
complement-mediated cytolytic destruction of those cells bearing the latent viral
protein.

Those of skill in the art readily understand how to apply the techniques
developed for imuunotherapy discussed herein to compositions which do not

25   include an antibody *per se*, but have the same antigen recognition properties of
an antibody. For example, peptides binding an "antigen" which are developed
de novo using combinatorial methods can be used to replace the "antibody" in
the immunotherapy regimes. These could be coupled to a radioisotope or toxin,
for example, for delivery to the target cell, as described below with respect to

30   the antibodies.

Coupling of Diagnostic or Cytotoxic Agents to Antibodies

The diagnostic or cytotoxic agents can be coupled either directly or
indirectly to the antibodies. Indirect coupling is typically via a spacer moiety.
These spacer moieties, in turn, can be either insoluble or soluble (Diener, et al.,

5 <u>Science</u>, 231:148 (1986)) and can be selected to enable release of the agent from the antibody molecule at the target site.

Some radioisotopes can be attached directly to the antibody; others require an indirect form of attachment. The radioisotopes ^{125}I, ^{131}I, ^{99m}Tc, ^{186}Re and ^{188}Re can be covalently bound to proteins (including antibodies) through

10 amino acid functional groups. For radioactive iodine it is usually through the phenolic group found on tyrosine.

There are numerous methods to accomplish coupling: chloramine-T (Greenwood, et al. <u>Biochem J</u>. 89: 114-123 (1963)); and Iodogen (Salacinski, et al. <u>Anal. Biochem.</u> 117: 136-146 (1981)). Tc and Re can be covalently bound

15 through the sulfhydryl group of cysteine (Griffiths, et al. <u>Cancer Res.</u> 51: 4594-4602 (1991)).

For *in vivo* diagnosis radioisotopes may be bound to immunoglobin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which

20 exist as metallic ions to immunoglobins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules.

The targeting molecule (for example, the antibody), and the agent can be linked in several ways. If the hybrid molecule is produced by expression of a

25 fused gene, a peptide bond serves as the link between the cytotoxin and the antibody or antibody fragment. Alternatively, the toxin and the binding ligand can be produced separately and later coupled by means of a non-peptide covalent bond. For example, the covalent linkage may take the form of a disulfide bond. In this case, the DNA encoding the antibody can be engineered

30 to contain an extra cysteine codon. The cysteine must be positioned so as not to interfere with the binding activity of the molecule. The toxin molecule must be derivatized with a sulfhydryl group reactive with the cysteine of the modified antibody. In the case of a peptide toxin this can be accomplished by inserting a cysteine codon into the DNA sequence encoding the toxin. Alternatively, a

35 sulfhydryl group, either by itself or as part of a cysteine residue, can be

5   introduced using solid phase polypeptide techniques. For example, the

introduction of sulfhydryl groups into peptides is described by Hiskey (Peptides

3:137 (1981)). The introduction of sulfhydryl groups into proteins is described

in Maasen et al. (Eur. J. Biochem. 134:32 (1983)). Once the correct sulfhydryl

groups are present, the cytotoxin and antibody are purified, both sulfur groups

10   are reduced; cytotoxin and ligand are mixed; (in a ratio of about 1:5 to 1:20) and

disulfide bond formation is allowed to proceed to completion (generally 20 to 30

minutes) at room temperature. The mixture is then dialyzed against phosphate

buffered saline or chromatographed in a resin such as Sephadex to remove

unreacted ligand and toxin molecules.

15   Numerous types of cytotoxic compounds can be joined to proteins

through the use of a reactive group on the cytotoxic compound or through the

use of a cross-linking agent. A common reactive group that will form a stable

covalent bond *in vivo* with an amine is isothiocyanate (Means, et al. Chemical

modifications of proteins (Holden-Day, San Francisco 1971) pp. 105-110). This

20   group preferentially reacts with the ε-amine group of lysine. Maleimide is a

commonly used reactive group to form a stable *in vivo* covalent bond with the

sulfhydryl group on cysteine (Ji, T.H., Methods Enzymol 91: 580-609 (1983)).

Monoclonal antibodies are incapable of forming covalent bonds with radiometal

ions, but they can be attached to the antibody indirectly through the use of

25   chelating agents that are covalently linked to the antibodies. Chelating agents

can be attached through amines (Meares, C.F., et al., Anal. Biochem. 142:68-78

(1984)) and sulfhydral groups (Koyama, et al., Chem. Abstr. 120:217262t

(1994)) of amino acid residues and also through carbohydrate groups (Rodwell,

J.D., et al., Proc. Natl. Acad. Sci. 83:2632-2636 (1986); Quadri, S.M., et al.,

30   Nucl. Med. Biol. 20:559-570 (1993)). Since these chelating agents contain two

types of functional groups, one to bind metal ions and the other to joining the

chelate to the antibody, they are commonly referred as bifunctional chelating

agents (Sundberg, M.N., et al., Nature 250:587-588 (1974)).

         Crosslinking agents have two reactive functional groups and are

35   classified as being homo or heterobifunctional. Examples of homobifunctional

5   crosslinking agents include bismaleimidohexane (BMH) which is reactive with

sulfhydryl groups (Chen, L.L., et al. <u>J Biol Chem</u> 266: 18237-18243 (1991) and

ethylene glycolbis[succinimidylsucciate] EGS which is reactive with amino

groups (Browning, J., et al., <u>J. Immunol.</u> 143: 1859-1867 (1989)). An example

of a heterobifunctional crosslinker is -maleimidobenzoyl-N-hydroxysuccinimide

10   ester (MBS) (Myers, DE et al. <u>J. Immunol. Meth.</u>121(1): 129-142 (1989)).

These methodologies are simple and are commonly employed.

Cytotoxic Agents

Toxins and substances which elicit the host to attack the tumor cells, as

well as synthetic or natural chemotherapeutic drugs (Halpern, et al., <u>J. Nucl.</u>

15   <u>Med.</u> 29:1688-1696 (1988); Quadri, et al., <u>Nucl. Med. Biol.</u> 20:559-570 (1993);

Wang, et al., <u>Radiat. Res.</u> 141:292-302 (1995)), oligonucleotides (Mujoo, et al.,

<u>Oncogene</u> 12:1617-1623 (1996)), cytokines (Markman, <u>Semin. Oncol.</u> 18:248-

254 (1991); Dedrick, et al., <u>Cancer. Treat Rep.</u> 62:1-11 (1978)), and radioactive

colloids (Rowlinson, et al., <u>Cancer Res.</u> 47:6528-6531 (1987)), can be

20   conjugated to the antibodies using standard chemical techniques, or in some

cases, using recombinant technology, for example, fusion proteins. The

antibodies can also be coupled to a signal protein that induces apoptosis (or

programmed cell death).

Toxins are poisonous substances produced by plants, animals, or

25   microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a

substance produced by *Corynebacterium diphtheria* which can be used

therapeutically. The toxic alpha component can be bound to the antibody and

used for site specific delivery to a cell harboring a latent virus, such as EBV.

Diphtheria toxin, whose sequence is known, and hybrid molecules thereof, are

30   described in detail in U.S. Patent No. 4,675,382 to Murphy.

Lectins are proteins, usually isolated from plant material, which bind to

specific sugar moieties. Ricin is a toxic lectin which has been used

immunotherapeutically. This is accomplished by binding the alpha-peptide

chain of ricin, which is responsible for toxicity, to the antibody molecule to

35   enable site specific delivery of the toxic effect. Other useful toxins include

5   cholera toxin, Shiga-like toxin (SLT-I, SLT-II, SLT-IIV), LT toxin, C3 toxin,

Shiga toxin, pertussis toxin, tetanus toxin, Pseudomonas exotoxin, alorin,

saporin, modeccin, and gelanin. See also Hoch, D.H., et al., Proc. Natl. Acad.

Sci. USA 82:1692-6 (1985); Colombatti, M., et al., J. Biol. Chem. 261:3030-5

(1986); Deleers et al., FEBS Lett., 160:82-6 (1983), Hwang, J., et al. (Cell

10   48:129-36 (1987)); and Gray, G.L., et al. (Proc. Natl. Acad. Sci. USA 81:2645-9

(1984)).

Radioisotopes are small and well characterized, and can be used as

diagnostics and followed after administration using standard non-invasive

radioimaging techniques. Certain radioisotopes may be more preferable than

15   others depending on such factors as viral distribution as well as isotype stability

and emission characteristics. In general, alpha and beta particle-emitting

radioisotopes are preferred in immunotherapy. Preferred are short range, high

energy alpha emitters such as ^{212}Bi. Examples of radioisotopes which can be

bound to the disclosed antibodies for therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y,

20   ^{67}Cu, ^{212}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, and ^{188}Re.

As radioisotopes decay, they emit characteristic photons or particles or

both. Photons, commonly referred to as gamma rays, are penetrating. If their

energy level is high enough, they can travel through the body and be detected by

diagnostic instrumentation. Radioisotopes that emit photons can be attached to

25   an antibody and used for diagnostic imaging. This application is termed

radioimmunoscintigraphy (RIS). The shorter the distance between the antigen

and the target, the shorter the required range of emission of the radioisotope.

Auger electrons have a very short path length (5-10 nm) and need to be

internalized to be cytotoxic (Adelstein, et al., Nucl. Med. Biol. 14:165-169

30   (1987)). Only antibodies that are internalized after binding to a cell should be

considered for radioisotopes that emit Auger electrons. Alpha particles need to

be close to a cell (within 3-4 cell diameters) to be effective (Vriesendorp, et al.,

Radioimmunoglobulin therapy. In: High Dose Cancer Therapy. Armitage, et

al. (eds). (Williams & Wilkins, Baltimore, MD 1992) pp. 84-123). Both Auger

5   electrons and alpha emitters have high selectivity because their short-range
emission will not irradiate neighboring normal cells.

The radiometals ^{111}In and ^{90}Y are, respectively, pure γ- and pure β-
emitters. Iodine-125, the most commonly used emitter of Auger electrons, has a
half-life of 60 days and frequently is released by the immunoconjugate *in vivo*

10   (dehalogenation) (Vriesendorp, et al., 1992). The most commonly considered
alpha emitters for clinical use, astatine-211 and bismuth-212, have short half-
lives (7.2 h and 1.0 h, respectively) and decay into radioactive isotopes, that may
not be retained by the immunoconjugate after the first alpha emission (Wilbur,
Antibiot. Immunoconjug. Radiopharm. 4:85-97 (1991)).

15   The antibodies can also be labeled with a paramagnetic isotope for
purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or
electron spin resonance (ESR). In general, any conventional method for
visualizing diagnostic imaging can be utilized. Usually gamma and positron
emitting radioisotopes are used for camera imaging and paramagnetic isotopes

20   for MRI.

Vaccines

Vaccines to induce a reaction to the latent viral proteins, will typically
consist of the viral protein, most preferably an extracellular portion thereof, as
described above, alone or in combination with an adjuvant. Numerous vaccine

25   formulations are known to those skilled in the art.

As discussed above, EBV produces cells expressing LMP-2A, which are
latently infected cells. LMP-2A peptides, or the entire protein, can be obtained
by isolating the naturally occurring protein, or more preferably, engineered
peptides can either be made through synthetic mechanisms or through

30   recombinant biotechnology techniques. Peptides of up to about forty amino
acids, more preferably between four and twenty-five amino acids, most
preferably between four and eight amino acids, can be synthesized using any
one of the methods known to those skilled in the art. Immunogenic fusion
protein derivatives can be made by fusing a polypeptide sufficiently large to

35   confer immunogenicity to the target sequence by cross-linking *in vitro* or by

5   recombinant cell culture transformed with DNA encoding the fusion. For
    example, chimeric proteins consisting of multiple epitopes from one or more
    latent viral proteins can be used to generate a more immunogenic molecule for
    administration as a vaccine.

    Peptide molecules can be generated in a variety of ways well known to
10 those of ordinary skill in the art. An example is the solid phase synthesis
    described by J. Merrifield (J. Am. Chem. Soc. 85, 2149 (1964)), used in U.S.
    Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a
    protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of
    a peptide starting from the C-terminus of the peptide. Other methods of
15 synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891. These
    methods can be used to synthesize peptides having identical sequence to the
    proteins described herein, or substitutions or additions of amino acids, which
    can be screened for activity as described above.

    Recombinant DNA technology can be used to generate large numbers of
20 peptides. Indeed, one approach uses what is called a phage display library. Here
    the DNA is transcribed into mRNA which is then translated to become part of
    the minor pili protein. By randomly varying the critical part of the DNA, a large
    number of peptides are prepared, which can include all of the theoretically
    possible peptides. In addition to their use in vaccines, the peptides can be used to
25 evaluate antigenicity or other interactions between ligands.

    Pharmaceutically Acceptable Carriers

    The compositions, including antibodies, can be used therapeutically in
    combination with a pharmaceutically acceptable carrier.

    Pharmaceutical carriers are known to those skilled in the art. These most
30 typically would be standard carriers for administration of vaccines to humans,
    including solutions such as sterile water, saline, and buffered solutions at
    physiological pH. Other ingredients include excipients, carriers, thickeners,
    diluents, buffers, preservatives, and surface active.

    Preparations for parenteral administration include sterile aqueous or non-
35 aqueous solutions, suspensions, and emulsions. Examples of non-aqueous

5   solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive

oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include

water, alcoholic/aqueous solutions, emulsions or suspensions, including saline

and buffered media. Parenteral vehicles include sodium chloride solution,

Ringer's dextrose, dextrose and sodium chloride, and lactated Ringer's.

10   Preservatives and other additives may also be present such as, for example,

antimicrobials, anti-oxidants, chelating agents, and inert gases.

The immunogenic peptides can also be administered in a viral vector,

such as an adenoviral vector, which infects cells, presents the latent viral protein

epitopes on the surfaces of the infected cells and thereby elicits the immune

15   response against the virus, i.e., EBV. Suitable adenoviral vector and vaccinia

virus systems are described by Ranier, et al., J. Virol. 73(12):10416-10425

(1999) and Stewart, et al., Vaccine 17:152-157 (1999), respectively.

Alternatively, the immunogenic peptides can be administered as DNA in a

plasmid, which can be administered directly as naked DNA, in cells, and/or

20   encapsulated in a polymer such as microspheres or hydrogels. These techniques

are well known to those skilled in the art. Charo, et al., J. Immunol. 163:5913-

5919 (1999) describes a suitable plasmid delivery system. Polymeric carriers

and other delivery systems are described, for example, in U.S. Patent Nos.

6,133,026, 6,080,728, 6,048,736, 5,985,573, 5,869,103, and 5,783,567.

25   **Substrates for Removal of Infected Cells**

The latently infected cells, once identified by the compositions herein,

can be separated from the remainder of the cells following procedures such as

bone marrow or solid organ transplants, or by standard techniques such as

plasmapheresis. These tend not to be selective, however.

30   More preferably, infected cells are removed with a selective process.

For example, antibody can be covalently bound to a solid phase and employed

as a composition for separation of latently infected cells from the remainder of

cells. In a preferred embodiment, the patient's blood is passed through an

extracorporeal reactor or filter having the anti-viral antibodies immobilized

35   thereon or therein. Typical filters are of the type used for kidney dialysis and

5   can be attached to the patient in the same manner. Conventional coupling

techniques, such as those described above, are used to secure the antibodies to

the nitrocellulose membranes in the dialyser units.

II.   Methods of Diagnosis and Treatment

There are a number of uses for the disclosed compositions, for example,

10   in diagnostic assays and kits, vaccine preparations, and a variety of therapies.

The destruction of cells harboring latent virus should reduce the effects

of the virus on the host and moderate or prevent any subsequent diseases that are

dependent on the presence of the DNA virus. For example, diseases such as

lupus (or any of the other diseases thought to be caused by EBV) which are

15   believed to require the continued presence of the EBV infection in order to

maintain the disease process, could thereby be moderated by the reduction in

EBV infected cells.

Therapies

Diseases to be Treated

20   Latent viral infections have been correlated with certain diseases, for

example, EBV infection is correlated with autoimmune disease and cancer, as

demonstrated by a number of published studies.

For example, a Japanese group found a high frequency of antibodies

against Epstein-Barr virus Nuclear Antigens 2 and 3 in lupus patient sera (an

25   autoimmune disease), compared to normal controls (Kitagawa, H., Et al.

Immunol. Lett. 17:249-252 (1988)). Another Japanese group found higher

levels of antibody directed against a membrane antigen from Epstein-Barr virus

in lupus (and rheumatoid arthritis) sera than in controls (Yokochi, T. et al. J.

Rheumatol. 16:1029-1032 (1989)). Similarly, an Australian group found a

30   modest increase in antibodies against early antigens (Sculley, D.G., et al. J. Gen.

Virol. 67:2253-2258 (1986)). An Italian group has shown that the affinity

purified antibodies from the 95-119 region of Sm D from lupus patients bind the

Epstein-Barr virus Nuclear Antigen-1 between amino acids 35 and 58

(Sabbatini, A., et al. Eur. J. Immunol. 23:1146-1152 (1993)). The most recent

35   contribution to this question uses both molecular methods to detect Epstein-Barr

5   DNA and serologic methods to detect antibodies to Epstein-Barr virus (Tsai, Y.

et al. Int. Arch. Allergy Immunol. 106:235-240 (1995)). These studies show no

significant differences between lupus patients and controls.

Morshed and colleagues published data showing an increased level of

Epstein-Barr virus DNA in patients with primary biliary cirrhosis compared to

10   controls from peripheral blood mononuclear cells, saliva, and fixed liver tissue

(Morshed, S.A. et al. Gastroenterol. Jpn. 27:751-758 (1992)). The nuclear dot

antigen is an autoantigen bound by autoantibody found in a few sera from

patients with primary biliary cirrhosis. This autoantibody is uncommonly found

in lupus and rheumatoid arthritis sera. Analysis of the epitopes of the nuclear

15   dot antigen has revealed two epitopes which have homology with Epstein-Barr

virus protein sequences (Xie, K. and Snyder, M. Proc. Natl. Acad. Sci. 92:1639-

1643 (1995)).

Evidence consistent with a relationship between lupus and EBV is the

following: The anti-Sm response may develop as a consequence of molecular

20   mimicry between PPPGRRP of EBNA-1 and PPPGMRPP of the Sm B/B'

autoantigen of the spliceosome (James JA and Harley JB, J. Immunol.

148:2074-79 (1992); James JA, et al. J. Exp. Med. 181:453-61 (1995)). EBV

infection is associated with lupus in both children and adults (James JA, et al. J.

Clin. Inv. 100:3019-26; James JA and Harley JB Arthritis Rheum., in press

25   (1999)). The immune response against EBV is qualitatively different in lupus

patients than it is in adults (James, J.A., et al., Arthritis Rheum., 41:S308

(1998)). EBV infection tends to precede lupus in affected individuals.

Other autoimmune diseases, including both rheumatoid arthritis and

Sjogren's syndrome, have been explored for a possible relationship to Epstein-

30   Barr virus. See Fox R.I., et al. J. Rheumatol. 19:18-24 (1992). The evidence

which they conclude supports a role for Epstein-Barr virus in rheumatoid

arthritis includes: similarity between synovial and viral antigens, higher levels of

antibodies against the Epstein-Barr virus Nuclear Antigens 1 and 3, and the

lower ability of lymphocytes to prevent the outgrowth of autologous, Epstein-

5   Barr virus infected lymphocytes (Fox, R.I. Current Opin. Rheum. 7:409-416
(1995)).

Table 1. Frequency of EBV-related, RAP or RANA antibodies by
precipitation in RA patients and controls.

| | RA | | controls | | | |
Author (year)	#pos/total	%	#pos/total	%	odds ratio	p-value
G Dalldorf (1969)[1]	6/ 21	29%	2/106	1.9%	20.8	0.00001
MA Alspaugh (1976)[2]	69/141	49%	6/ 71	8%	10.41	<0.00001
MA Catalano (1979)[3]	44/ 47	94%	12/ 48	25%	44	<0.00001
PB Ferrel (1981)[4]	62/ 87	71%	3/ 53	6%	69	<0.00001
MA Alspaugh (1981)[5]	44/ 61	72%	1/ 16	6%	39	<0.00001
PJW Venables (1981)[6]	86/100	86%	50/ 93	54%	5.3	<0.00001
DA Bell (1984)[7]	13/ 31	42%	3/ 26	12%	5.5	0.02
K Nakabayashi[8] (1985)	24/ 40	60%	2/ 30	7%	21	<0.00001

[1]Dalldorf, G., et al., J. Amer. Med. Assn. 208:1365-8 (1969)
20 [2]Alsbaugh, M.A. & Tan, E.M., Arthritis Rheum. 19:711-9 (1976)
[3]Catalano, M.A., et al., Proc. Natl. Acad. Sci USA 76:5825-8 (1979)
[4]Ferrel, P.B., et al., J. Clin. Invest. 67:681-7 (1981)
[5]Alsbaugh, M.A., et al., J. Clin. Invest. 67:1134-40 (1981)
[6]Venables, P.J.W., et al., Arthritis Rheum. 24:1459-68 (1981)
25 [7]Bell, D.A. & Alsbaugh, M.A. J. Rheumatol. 11:277-81 (1984)
[8]Nakabayashi, K., et al., Rheumatol. Int. 5:61-7 (1985)

Table 2. Frequency of anti-RANA antibodies by immunofluoresence in RA
patients and controls.

| | RA | | controls | | | |
Author (year)	#pos/total	%	#pos/total	%	odds ratio	p-value
EM Tan (1979)[1]	59/ 90	67%	6/71	8%	21	<0.00001
KC Ng (1980)[2]	116/124	94%	8/50	16%	76	<0.00001

35 [1]Tan, E.M. Rev. Inf. Dis. 1:997-1006 (1979)
[2]Ng, K.C., et al., Lancet 1(8166):447-9 (1980)

5 **Table 3. Frequency of anti-EBNA-1 antibodies by immunofluoresence in RA patients and controls.**

	RA		controls			
Author (year)	#pos/total	%	#pos/total	%	odds ratio	p-value
MA Catalano (1979)[1]	49/50	98%	41/47	87%	7.2	0.05
KC Ng (1980)[2]	55/64	86%	36/50	72%	2.4	>0.05
PB Ferrel (1981)[3]	77/80	97%	45/51	89%	3.4	>0.05
A Kahan (1985)[4]	47/47	100%	14/14	100%	1.0	1.0
M Musiani (1987)[5]	19/35	54%	6/40	15%	6.7	<0.0001
T Yokochi (1989)[6]	18/22	82%	2/14	14%	27	<0.00001

15 [1]Catalano, M.A., et al., Proc. Natl. Acad. Sci. USA 76:5825-8 (1979)
[2]Ng, K.C., et al., Lancet 1(8166):447-9 (1980)
[3]Ferrel, P.B., et al., J. Clin. Invest. 67:681-7 (1981)
[4]Kahan, A:, et al., Arthritis Rheum. 28:961-70 (1985)
[5]Musaini, M., et al. Ann. Rheum. Dis. 46:837-42 (1987)
20 [6]Yokochi, T., et al., J. Rheumatol. 16:1029-32 (1989)

It has been proposed that Epstein-Barr virus is an etiologic agent for Sjogren's syndrome (Whittingham, S., et al. Med. Hypothesis 22:373-386 (1987)). They postulate that the combined effect of Epstein-Barr virus infection 25 and autoimmunity leads to Sjogren's syndrome.

A higher level and frequency of Epstein-Barr virus, as well as other viruses, are found in salivary gland epithelium and gland tissue of patients with Sjogren's Syndrome (Fox, R.I. et al. J. Immunol 137:3162-3168 (1986)); (Fox, R.I. Current Opin. Rheum. 7:409-416 (1995)). Evidence for Epstein-Barr virus 30 in 80% of the lacrimal gland specimens from Sjogren's syndrome patients and in none of the controls was reported by Pflugfelder, S.A. et al Ophthalmology 97:976-984 (1990); and Pflugfelder, S.A. et al. Am. J. Pathol. 143:49-64 (1993). Higher levels of hybridization between an Epstein-Barr virus DNA probe and the nuclei of salivary gland epithelial cells in Sjogren's syndrome than in 35 controls was reported by Karameris, A. et al. Clin. Exp. Rheum. 10:327-332 (1992).

5   An increase in the Epstein-Barr virus production by B cells in patients
with Sjogren's syndrome was described by Tateishi, M. et al. Arthritis Rhuem.
36:827-835 (1993). A minor increase in antibody levels against Epstein-Barr
virus Nuclear Antigen-2 domains in Sjogren's syndrome compared to controls
was published by Inoue, N. et al. J. Infect. Dis. 164;22-28 (1991). A modest

10   elevation of anti-Epstein-Barr Nuclear antigen, anti-Early Antigen and anti-
Epstein-Barr virus Viral Capsid Antigen (all measured by immunofluorescence)
was described by Toda, I., et al. Sjogren's syndrome (SS) and Epstein-Barr virus
(EBV) reactivation. In Lacrimal Gland, Tear Film, and Dry Eye Syndrome.
D.A. Sullivan, ed. pp 647-650 (Plenum Press, New York 1994).

15   Others, however, found no such relationship and concluded that the
frequency of Epstein-Barr virus DNA in salivary biopsy specimens was no
different in patients with Sjogren's syndrome when compared with normal
(Venables, P.J.W., et al. Clin. Exp. Immunol. 75:359-364 (1989); Venables,
P.J.W., et al. J. Autoimmunity 2:439-438 (1989); Deacon, L.M., et al. Am J.

20   Med. 92:453-454 (1992); Venables, P.J.W. et al. Clin. Exp. Immunol. 75:359-
364 (1989); Maitland (Maitland, N.J. Am. J. Med. 96:97 (1994); Deacon, E.M.,
et al. J. Pathol. 163:351-360 (1991); Mariette, X., et al. Am. J. Med. 90:286-294
(1991).

        An example of double infection with Epstein-Barr virus and another

25   virus is found in a cell line isolated from a patient with apparent multiple
sclerosis (Haahr, S. et al. Ann. N. Y. Acad. Sci. 724:148-156 (1996)). The
increased prevalence of seroconversion among multiple sclerosis patients,
relative to controls, has led to the suggestion that Epstein-Barr virus may be an
etiologic agent in multiple sclerosis (Sumaya, C.V. et al. Ann. Neurol. 17:371-

30   377 (1985); Bray, P.F., et al. Arch. Neurol. 40:406-408 (1983); Larsen. P.D., et
al. Neurology 35:435-438 (1985); Warner, H.B. and Carp. R.I. Med. Hypothesis
25:93-97 (1988); Bray, P.F. et al. Neurology (1992)).

        It is therefore believed that agents specifically targeted to latent viral
proteins, especially EBV latent proteins, may be effective to reduce or treat

5   diseases including autoimmune diseases such as SLE, Sjogren's Syndrome, rheumatoid arthritis, and Multiple Sclerosis.

Methods of Administration and Dosages

Therapy can be by inducing an immune response to the latent virus (vaccine), killing of the infected cells, or removal of infected cells. The

10   antibody conjugated to a cytotoxic agent can also be used for immunotherapy to kill cells infected with a virus that is at least partly in the latent stage of its life cycle and is expressing the proteins of latency with epitopes reactive with the disclosed antibodies. Effective dosages of antibody conjugate will vary based upon affinity, selectivity and concentration of the antibody.

15   The dosage ranges for the administration of the antibody conjugates are those large enough to ameliorate the symptoms of the immune response mediated disorder, but low enough to avoid adverse side effects, such as unwanted cross-reactions or anaphylactic reactions. Generally, the dosage will vary with the age, condition, and extent of the disease in the patient and can be

20   determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary from one to multiple doses administered daily, for one to several days. Generally, when the antibodies of the invention are administered conjugated with therapeutic agents lower dosages, as compared those used for *in vivo*

25   immunodiagnostic imaging, can be used.

In a preferred embodiment, the vaccine is administered in a dosage and on a schedule which elicits a response capable of either blocking primary EBV infection or significantly reducing the EBV load during primary infection, to avert clinical symptoms.

30   *Vaccination*

A protocol for vaccination to induce an immune response to the infected cells is designed based on standard techniques. Efficacy can be determined based on measurements of antibody titers to the latent viral proteins and using diagnostic techniques to determine the presence and/or number of infected cells

35   remaining after treatment. Vaccination protocols range from a single

5   immunization to multiple boosters. Priming vaccinations followed by boosters

may be required, or it may be necessary to utilize a prolonged treatment protocol

with low quantities of antigen, as is done with the treatment of allergies.

Diagnostic Uses

Labelled antibody conjugates can be used to confirm and/or quantitate

10   the presence of a latent viral infection. It is also possible to determine whether a

particular therapeutic regimen aimed at reducing the viral infection is effective.

The antibodies specifically binding to the latent viral antigens present on

the surface of the cells infected with the virus can be used in assays with either

liquid phase or solid phase carriers and with any of a variety of labels, in either

15   competitive or noncompetitive assays and in either a direct or indirect format.

Examples of such immunoassays are the radioimmunoassay (RIA) and the

sandwich (immunometric) assay. Immunoassays can be run in either the

forward, reverse, or simultaneous modes, including immunohistochemical

assays on physiological samples.

20   The extracellular portion of the targeted latent viral antigen is presented

on the surfaces of cells. These cells can therefore be detected by the disclosed

antibodies in biological fluids and tissues. Any sample containing a detectable

amount of the extracellular portion of LMP-2A or a cell expressing the

extracellular portion of LMP-2A can be used. Normally, a sample is a liquid

25   such as saliva, cerebrospinal fluid, blood, or serum or a solid or semi-solid such

as tissues.

In using the disclosed antibodies for the *in vivo* detection of the latent

viral antigen, the detectably labeled monoclonal antibody is given in a dose

which is sufficient to enable detection of cells which are latently infected with

30   virus.

It is desirable that the detectably labeled antibody be rapidly cleared

from the circulatory system in order to give the best target-to-background signal

ratio. Typically, the dosage of antibody will vary from about 0.01 mg/m^2 to

about 20 mg/m^2, preferably about 0.1 mg/m^2 to about 10 mg/m^2.

5   For *in vivo* diagnostic imaging using a radioisotype, the type of detection

instrument available is a major factor in selecting a given radioisotope. The

radioisotope chosen must have a type of decay which is detectable for a given

type of instrument. Still another important factor in selecting a radioisotope for

in vivo diagnosis is that the half-life of the radioisotope be long enough so that it

10   is still detectable at the time of maximum uptake by the target, but short enough

so that deleterious radiation with respect to the host is minimized. Ideally, a

radioisotope used for *in vivo* imaging will lack a particle emission, but produce a

large number of photons in the 140-250 keV range, which may be readily

detected by conventional gamma cameras.

15   A preferred treatment is the use of viral proteins to vaccinate a patient against

the virus. Peptides used as vaccines are preferably administered

intramuscularly or subcutaneously. The dose, schedule of doses and route of

administration may be varied, whether oral, nasal, vaginal, rectal, extraocular,

intramuscular, intracutaneous, subcutaneous, or intravenous, to avoid

20   autoimmunity and, yet, to achieve immunity from Epstein-Barr virus infection.

The response to the unmodified vaccine may be further influenced by its

composition. The particular adjuvant employed (its concentration, dose and

physical state), concentration of the virus in the vaccine, and treatment of the

unmodified vaccine with physical environmental changes, for example,

25   temperature and pressure, the particular buffer, and the particular preservative(s)

(if any) will be selected to reduce the likelihood of developing an autoimmune

disorder, for example using the animal strains discussed below. This same or

different vaccine may be useful in reducing or eliminating the effect of an

existing latent or active Epstein-Barr virus infection upon autoimmunity.

30   **Methods for Administration**

The pharmaceutical composition may be administered in a number of ways

depending on whether local or systemic treatment is desired, and on the area to be

treated. Administration may be topically (including ophthalmically, vaginally,

rectally, intranasally), orally, by inhalation, or parenterally, for example by

35   intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The

5   disclosed antibodies can be administered intravenously, intraperitoneally,

intramuscularly, subcutaneously, intracavity, or transdermally.

The present invention will be further understood by reference to the

following non-limiting examples.

Example 1:   Preparation of anti-latent EBV antibodies.

10   To generate an *E. coli* LMP-2A expression plasmid, a 1,029 bp SalI/NsiI

fragment was removed from the LMP-2A cDNA clone (obtained from Dr. Mike

Kurilla, formerly from the Department of Pathology, University of Virginia

Health Sciences Center) (Figure 1). This fragment of LMP-2A cDNA

corresponds to bp 789-1817 of the GenBank LMP-2A entry (Accession

15   #M24212) and encodes amino acids 259-497 of LMP-2A as well as some of the

3' untranslated sequence. The fragment was ligated into SalII/PstI digested

pMal-C2 (New England Biolabs, Beverely, MA). The resulting construct

encodes a maltose binding protein (MBP) LMP-2A fusion protein (construct

#1). Separating the maltose binding protein and LMP-2A is a run of 20

20   arginines and a Factor Xa cleavage site. The Factor Xa cleavage site allows the

LMP-2A peptide fragment to be separated and isolated from the maltose

binding protein moiety.

A second construct was generated by cloning a 1,760 bp BamHI/NsiI

fragment from the LMP-2A cDNA clone and ligated into BamHI/PstI digested

25   pMal-C2. To maintain the proper open reading frame the new plasmid was

digested with BamHI and XmnI, blunt-ended with the Klenow DNA

polymerase I fragment, and re-ligated. The resulting plasmid encodes nearly

the entire full-length LMP-2A protein (a.a. 18-498). In fact, amino acids 1-14

encode the signal sequence. Thus, this construct encodes all but the first four

30   amino acids of the mature peptide. The resulting construct encodes a MBP-full

length LMP-2A fusion protein. *E. coli* cells transfected with the LMP-2A fusion

protein construct appeared to express a full-length protein. This was based on

the presence of a 93,000 molecular weight protein on SDS-PAGE and Western

blot detection using rabbit anti-MBP polyclonal sera. However, over 90% of

35   the protein expressed from this construct appeared to be native maltose binding

5 protein (from affinity purification with an amylose resin column). This could
have been due to proteolysis, instability of the fusion protein, or premature
translational termination. Because of the instability of the full-length LMP-2A
fusion protein anti-LMP-2A antibodies were generated using the more stable
and higher expressed truncated LMP-2A fusion protein.

10   A third LMP-2A construct was generated by cloning a SalI/HindIII
fragment from the pMal-truncated LMP-2A fusion protein plasmid (construct
#1). This fragment was cloned into the pQE9 expression vector (Qiagen). This
vector expresses the truncated LMP-2A peptide with an N-terminal six His tag.
Which can be used for affinity purification on a nickel column. This fragment
15 has a molecular weight of 27,000 and the only additional amino acids are the
six N-terminal histidine residues.

     E. coli cells transfected with the truncated MBP-LMP-2A fusion protein
plasmid (construct #1) expressed the MBP-LMP-2A fusion protein (Figure 2).
This was based on the presence of a 69,000 molecular weight protein on SDS-
20 PAGE and Western blot detection using rabbit anti-MBP polyclonal sera. The
LMP-2A fusion protein encodes 212 amino acids of the transmembrane
domains and the C-terminal 27 amino acids, which are intracellular, as opposed
to the 351 amino acids in the mature protein.

     MBP-LMP2A fusion protein was isolated from E. coli cells that had been
25 induced by the addition of IPTG to 0.3 mM for 2 hours at 37 C. Cells were
harvested by centrifugation and lysed by sonication. The MBP-LMP2A fusion
protein was isolated from the cell lysate by affinity chromatography using
amylose resin. Bound MBP-LMP-2A was eluted from the column using 10
mM Maltose.

30 **Example 2:   Immunization of animals.**

     Immunizations were performed as follows: On day one, two New Zealand
White rabbits were immunized with 500 mcg recombinant MBP-LMP-2A
fusion protein (in 0.5 ml PBS) emulsified in 0.5 ml complete Freunds adjuvant
and injected by intraperitoneal and subcutaneous routes. On days 28, 56, and
35 100 the animals were boosted with another 500 mcg of antigen in incomplete

5   Freunds adjuvant. Pre-bleeds and weekly bleeds following the initial

immunization were collected by ear puncture to determine the titer of anti-

LMP-2A antibodies.

Western blot analysis showed that both the 55 kD wild-type LMP2A and

Factor Xa released truncated LMP-2A were bound by antibodies from the

10   animal immunized with MBP-truncated LMP-2A fusion protein (construct #1).

Sera from the immunized animal was diluted 1:100 and incubated with a

nictrocelluose membrane which had SDS-PAGE separated proteins

electrophoretically transferred to the membrane. The bound rabbit antibodies

were detected using goat anti-rabbit alkaline phosphatase conjugated antibodies.

15   These results showed that rabbits immunized with the MBP truncated LMP-2A

fusion protein generated antibodies that recognize LMP-2A.

Sera from immunized rabbits were also used in flow-cytometry

experiments. Cell lines tested included EBV-transformed human B cells and

murine L cells transiently transfected with a pCDNA-3 plasmid expressing the

20   full-length LMP-2A cDNA under control of the CMV promoter. One microliter

of rabbit sera was incubated with $1X10^6$ cells in 40 microliters PBS. Cells were

washed with PBS supplemented with to 1% BSA twice and incubated with

FITC labeled goat anti-rabbit IgG and washed twice again. Labeled cells were

visualized using a FacScan flow cytometer.

25   Two separate flowcytometry experiments showed that the rabbits

immunized with the MBP-truncated LMP-2A fusion protein generated

antibodies that recognize cells expressing LMP-2A. Pre-immune sera did not

bind to human B cells infected with EBV. However, sera from immunized

rabbits did. In addition, antibodies from the immunized sera did not bind to

30   mouse L cells transfected with pCDNA3 vector alone. However, antibodies

from the immunized sera did bind to mouse L cells transfected with the

pCNDA3 vector containing the LMP-2A cDNA. These results showed that at

least a population of the anti-LMP-2A antibodies recognized cells surface

expressed LMP-2A.

5   Proteins were prepared from the known sequence of LMP-2A and were

used to immunize rabbits. The results indicate that a variety of these antibodies

bind extracellular portions of LMP-2A. Cells that express LMP-2A are bound

by antibody to LMP-2A. Control cells do not express LMP-2A and are not

bound by the antibodies to LMP-2A.

10   Epstein-Barr virus, as well as most of the other human herpesviruses, is

limited to infection of only the human host. Non-primate animal species do not

have the necessary complement receptor function to allow EBV infection. Also,

other primates have their own variants of gamma herpes viruses. Since lower

animals are not infected with EBV, they are not normally exposed to LMP-2A

15   and no antibodies are usually made.

Balb/c mice were immunized with the same LMP-2A construct outlined

above (construct #1). Balb/c mice were also immunized with a 6-Histidine-

tagged LMP-2A (construct #3). These mice have mounted a significant immune

response to LMP-2A. Over 500 hybridomas were generated from mice

20   immunized with truncated LMP-2A and screened by ELISA for LMP-2A

specificity. Three clones were identified that contained the desired specificity

(bound LMP-2A fusion protein but not Maltose binding protein control).

This data is predictive of results with other latent proteins such as LMP-

2B. LMP-2B differs from LMP-2A in the cytoplasmic portion of the molecule.

25   Consequently, a similar availability of the extracellular portions of these two

molecules is predicted.